EXHIBIT 8

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Identification of a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of *Chlamydia psittaci*

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Abstract

While attempting to identify genes and their corresponding antigens that could be used to improve the current methods of diagnosing *Chlamydia psittaci* infection which causes enzootic abortion in ewes, two candidate clones were isolated from a λgt11 genomic DNA expression library of ovine abortion subtype (strain S26/3) *C. psittaci*. These clones contained fragments of a gene coding for a group of three chlamydial proteins of approximately 90 kDa which appeared as major immunogens by immunoblotting experiments, indicating their potential as diagnostic or possibly protective antigens. Southern blotting of S26/3 genomic DNA using the two clones as probes identified a family of three or four genes. These represent the first example of protein gene duplication reported in *Chlamydia*.

Keywords: *Chlamydia psittaci*; 90 kDa outer membrane protein; Immunoscreening; Affinity-purified antibody; Western blotting; Southern blotting

1. Introduction

A subtype ('Immunotype 1') of *Chlamydia psittaci* causes ovine enzootic abortion (OEA). The disease results in substantial economic losses in the UK [1] and the OEA agent can also cause abortion in women [2]. Some control has been achieved by vaccination [3] or antibiotic treatment but neither of these measures completely eliminates the disease [4,5]. Schemes to certify flocks free of the disease exist but surveillance by serodiagnosis alone has not been possible since there is currently no completely specific test. The problem of false positive values is thought to arise from widespread infection with a second chlamydial species, *C. pecorum* [6,7], which commonly infects the gut but has also been isolated from cases of polyarthritis and conjunctivitis [8,9].

Our research has focused on both trying to improve serodiagnosis [10] and attempting to develop a recombinant vaccine against OEA. Consequently, our interest centred on protein antigens which are specific to OEA *C. psittaci* and especially those located in the outer membrane which might be involved in protective immunity. Our attention has thus been drawn to proteins, with a size of around 90 kDa, which react strongly in immunoblotting experiments [8,11,12] and which are present in chlamy-

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dial outer membrane complexes (COMCs) [12,13]. We have previously shown that such COMCs can confer protective immunity when used in an experimental vaccine [14]. In this communication we report the first isolation of clones of the genes for the 90 kDa proteins and the finding that there is a family of such genes, the first example of protein gene duplication to be reported for *Chlamydia*.

2. Materials and methods

2.1. Immunoscreening of a λgt11 library

The OEA *C. psittaci* strain S26/3 used in this work has been previously described, as has the method for genomic DNA extraction [15]. A randomly sheared, size-selected genomic DNA library constructed in λgt11 from S26/3 DNA was immunoscreened by standard techniques with a pool of post-abortion sera from experimentally infected ewes. Plaques expressing recombinant fusion peptides were transferred to nitrocellulose membrane (Hybond-C Super; Amersham International) and blocked with 5% dried skimmed milk powder/Tris-buffered saline (TBS), pH 7.6 for 2 h. Membranes were incubated overnight with a 1/100 dilution of the pool of post-abortion sera, pre-absorbed with *E. coli* strain Y1090, in 0.1% Tween 20/5% normal donkey serum/TBS. Bound antibody was detected using horseradish peroxidase-conjugated donkey anti-sheep IgG (Sigma Chemical) with 0.67 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in 0.1 M Tris-HCl, pH 7.5/0.033% nickel chloride/0.0075% hydrogen peroxide as substrate. Positive phage clones were subjected to multiple rounds of immunoscreening until plaque purity was achieved.

2.2. Affinity purification of antibody on recombinant phage

Recombinant phage were plated onto a lawn of *E. coli* Y1090, and recombinant fusion protein expressed and transferred to nitrocellulose membrane as for immunoscreening. After blocking and incubating with sera the membrane was washed 6×5 min with 0.1% Tween 20/TBS and bound antibody eluted by two applications of 2 ml elution buffer (5 mM glycine, pH 2.3/0.5 M NaCl/0.2% Tween 20) for 3 min each. The resulting 4 ml of antibody solution was neutralised by the addition of 200 μl 1 M Tris-HCl, pH 7.4 and diluted 2-fold with 10% normal donkey serum in TBS.

2.3. Immunoblotting of EB extracts

C. psittaci S26/3 elementary bodies were purified from infected L929 cells as described previously [15] and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after boiling in sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Protein was transferred to nitrocellulose membrane by semi-dry blotting and incubated with affinity-purified antibody or post-abortion sera. Antibody binding was detected as described in Section 2.1.

2.4. Cloning and Southern blotting

Recombinant lambda DNA was produced by subjecting individual immunopositive plaques, picked

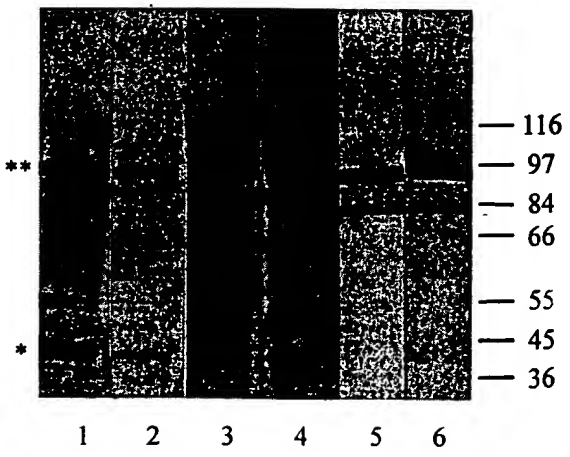

Fig. 1. Immunoblotting analysis of elementary body preparations. Whole EB preparations were subjected to SDS-PAGE on 7.5% gels under reducing conditions and immunoblotted with the pool of post-abortion sera used to screen the λgt11 library (lane 1); a pool of negative control sera from a flock free of chlamydial infection (lane 2); sera from two individual ewes, 3205 (lane 3) and 3210 (lane 4); and affinity-purified antibodies from clones 99 (lane 5) and 31 (lane 6). Molecular masses expressed in kDa are as indicated. **Indicates the 90 kDa protein triplet and *denotes the major outer membrane protein (MOMP).

A

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1   GTT GAT TAT ACA GGA AAG ATC GTC TTC TCA GGT GAA AAA TTA TCC GAT GAA GAA AAA GCA CGA GCG GAA AAC CTA GCT
    Val Asp Tyr Thr Gly Lys Ile Val Phe Ser Gly Glu Lys Leu Ser Asp Glu Glu Lys Ala Arg Ala Glu Asn Leu Ala

79  TCT ACT TTC AAC CAA CCC ATC ACA TTA TCA GCA GGA TCT CTT GTA CTT AAA GAT GGT GTA TCT GTA ACC GCA AAA CAA
    Ser Thr Phe Asn Gln Pro Ile Thr Leu Ser Ala Gly Ser Leu Val Leu Lys Asp Gly Val Ser Val Thr Ala Lys Gln

157 GTA ACG CAG GAA GCG GGA TCT ACC GTT GTC ATG GAT CTA GGG ACC ACA TTA CAG ACG CCT  216
    Val Thr Gln Glu Ala Gly Ser Thr Val Val Met Asp Leu Gly Thr Thr Leu Gln Thr Pro
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B

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1   GAA TTC GAG GTA AAA GAG ACT ACA TCA GGA GCA ATT TAT ACT TGC GAA GGT AAT GTG TGC ATC TCC TAT GCA GGG AAA
    Glu Phe Glu Val Lys Glu Thr Thr Ser Gly Ala Ile Tyr Thr Cys Glu Gly Asn Val Cys Ile Ser Tyr Ala Gly Lys

79  GAT TCT CCT CTA AAT AAA AGT TGT TTC TCA GAA ACT ACT GAA AAT CTT TCT TTC ATA GGA AAT GGT TAC ACC TTG TGT
    Asp Ser Pro Leu Asn Lys Ser Cys Phe Ser Glu Thr Thr Glu Asn Leu Ser Phe Ile Gly Asn Gly Tyr Thr Leu Cys

157 TTT GAT AAT ATT ACT ACA CAA TCT AGT CAC CCC GGA GCT ATT AGT GTT AGT GGT ACC AAT AAA ACC TTA GAC ATC TCA
    Phe Asp Asn Ile Thr Thr Gln Ser Ser His Pro Gly Ala Ile Ser Val Ser Gly Thr Asn Lys Thr Leu Asp Ile Ser

235 GGA TTT TCC TTA TTT TCA TGT GCC TAT TGC TGC CCT CCA GGA ACC ACC GGT TAC GGA GCT ATA CAG ACT AAA GGC ACC
    Gly Phe Ser Leu Phe Ser Cys Ala Tyr Cys Cys Pro Pro Gly Thr Thr Gly Tyr Gly Ala Ile Gln Thr Lys Gly Thr

313 ACA ACT TTA AAA GAT AAC TCT AGT CTT GTC TTC CAT AAA AAC TGT TCG ACA GCA GAG GGT GGC GCT ATT CAG TGT AAA
    Thr Thr Leu Lys Asp Asn Ser Ser Leu Val Phe His Lys Asn Cys Ser Thr Ala Glu Gly Gly Ala Ile Gln Cys Lys

391 TCA AGC AGC TCT ACT GCT GAG TTA AAG CTA GAA AAT AAT AAA AAT CTT GTT TTC TCA GAG AAT TC  455
    Ser Ser Ser Ser Thr Ala Glu Leu Lys Leu Glu Asn Asn Lys Asn Leu Val Phe Ser Glu Asn
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Fig. 2. Nucleotide sequence and protein translation of clone 31 (A) and 99 (B) DNA inserts. Both nucleotide sequences have been deposited in the GenBank database with accession numbers U59306 and U59307 for clones 31 and 99, respectively.

into 100 μl sterile water, to multiple freeze-thaw cycles and boiling for 5 min: 5 μl of the DNA solution was used per PCR reaction. PCR amplification of the DNA was performed using primers which flanked the *Eco*RI insertion site (primers 1218 and 1222; New England Biolabs, USA), and the Expand Long Template PCR System (Boehringer Mannheim).

PCR-derived insert DNA was cloned into the T-vector pGEM-T (Promega) and transformed, according to instructions supplied with the vector, by heat-shock into *E. coli* strain XL-1 Blue (Stratagene Ltd.) chemically competent cells prepared according to the method of Chung et al. [16]. Recombinant plasmid DNA was prepared using a QIAprep Spin Plasmid Miniprep Kit (QIAGEN Ltd.) and sequenced by the dideoxy chain termination method using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham International). Insert DNA was prepared by *Eco*RI digestion of purified plasmid DNA, gel purified using a Geneclean II Kit (Bio 101 Inc., CA, USA), labelled by the random-primed incorporation of digoxigenin (DIG)-labelled deoxyuridine triphosphate and used to probe Southern blots of S26/3. The labelling, hybridisation and detection of DIG-labelled probes were according to manufacturer's instructions (Boehringer Mannheim).

3. Results and discussion

Screening of the λgt11 S26/3 genomic DNA library with the pool of post-abortion sera identified two positive clones (31 and 99). Affinity-purified antibodies produced from these expressed recombinant lambda clones recognised a group of three proteins with molecular masses of approx. 90–95 kDa in the immunoblotting of S26/3 EBs (Fig. 1; lanes 5 and 6). This showed that the clones were derived from a gene(s) coding for the group of three proteins. These bands in the 90 kDa region are the same as the highly immunogenic proteins seen after immunoblotting EBs with individual sera from post-aborted ewes (for example, Fig. 1; lanes 3 and 4), or the pool of post-abortion antisera that was originally used to screen the λgt11 library (Fig. 1; lane 1). The

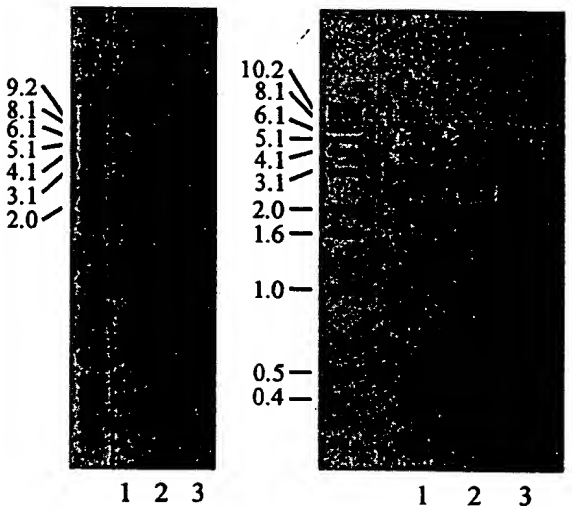

Fig. 3. Southern blotting analysis of ovine abortion strain S26/3 genomic DNA. Aliquots (250 ng) of S26/3 genomic DNA digested with *Eco*RI (lane 1), *Pst*I (lane 2) and *Xba*I (lane 3) were run on a 1% agarose gel in 0.5×TBE buffer, blotted onto Hybond-N nylon membrane and probed with digoxigenin-labelled clone 31 (A) and 99 (B) insert DNA (25 ng/ml) at 42°C in standard hybridisation buffer+50% formamide. The most stringent wash was in 1×SSC, 0.1% SDS at 42°C. Weak hybridisation signals are indicated by arrowheads. DNA size markers, expressed in kilobase pairs, are as indicated.

three proteins are almost certainly the same as the highly reactive triplet antigens bearing serotype 1- and subspecies-specific epitopes described by Souriau et al. [11] and Griffiths et al. [8]. Similarly, the highly immunoreactive 89 kDa protein described by Cevenini et al. [12], which was shown to be present in an outer-membrane MOMP-enriched sarkosyl-insoluble fraction, may also be one of these proteins.

In addition to detecting the 90 kDa proteins after immunoblotting proteins from whole EBs with the affinity-purified clone 31 and 99 antibodies other minor bands can be seen (Fig. 1; lanes 5 and 6) which may be degradation products or proteins related to the 90 kDa proteins.

Sub-cloning and sequencing revealed insert sizes for the two lambda clones, 31 and 99, of 216 and 455 bp, respectively (Fig. 2). The sequences consist entirely of open-reading frame (no stop codons present), do not overlap and code for expressed protein products of 7.5 and 16 kDa for clones 31 and 99, respectively. Searches in the GenBank/EMBL and SwissProt databases (Wisconsin GCG package, Seqnet facility, Daresbury laboratory, UK), and GSDB (Genome Sequence DataBase) database (On World Wide Web at URL http://www.tigr.org [17,18]) failed to find any related sequences.

Restriction endonuclease digestion and sequence analysis showed that neither clone 31 nor 99 contains *Eco*RI, *Pst*I or *Xba*I sites, but Southern blots of genomic DNA digested with these enzymes detected two clear bands indicating the presence of at least two related genes (Fig. 3). However, clone 99 probe additionally detected one *Pst*I and two *Eco*RI and *Xba*I fragments indicating the presence of at least three, and possibly four, genes (Fig. 3B). The relative intensities of the bands suggested that clones 31 and 99 originate from different genes: the 3.7 and 5.3 kb *Eco*RI, the 2.4 and 2.7 kb *Xba*I, and the 5.8 kb *Pst*I fragments detected with clone 31 probe (Fig. 3A) are only weakly detected by clone 99 probe (Fig. 3B).

This family of proteins is, to our knowledge, the first example of protein gene duplication that has been reported in *Chlamydia*. Although the function of these proteins has yet to be determined they may play a role in the entry of the bacterium into its target cell, similar to the adhesins in *Mycoplasma genitalium* [17] and *Listeria monocytogenes* [19] which are also reported as being part of multigene families. We are currently isolating and characterising these 90 kDa protein genes which, ultimately, will help improve our understanding of, and possibly provide a means of interfering with, the pathogenic mechanisms utilised by the organism.

Acknowledgments

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